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ALLELIC EXPRESSION ANALYSIS, AND ASSOCIATION WITH OA, OF COMMON VARIANTS IN SMAD3

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Purpose: In 2010 it was reported that the common SNP rs12901499, which is located in intron 1 of SMAD3, was associated with OA in European populations. The SMAD3-encoded protein is a critical component in the pathway that translocates the TGF-β signal to the cell nucleus in cartilage and in other articulating joint tissues. rs12901499 is not in high linkage disequilibrium with any common non-synonymous polymorphisms, making it unlikely that the OA association marked by this SNP is due to a change in sequence of the SMAD3 protein. Instead, influences on SMAD3 gene transcription or transcript stability, resulting in allelic expression imbalance, appear more likely the mechanism through which the genetic association is operating. In fact, many common complex traits are mediated by such cis-acting regulatory polymorphisms. In this study we therefore set out to assess whether the OA association to rs12901499 was marking allelic expression imbalance of SMAD3.
Methods: Using RNA extracted from the cartilage, fat pad or synovium of OA patients who had undergone elective joint replacement surgery, we assessed whether rs12901499 correlated with SMAD3 allelic expression by: 1) measuring SMAD3 expression by quantitative PCR and then stratifying the data by genotype at the SNP and 2) accurately discriminating and quantifying the mRNA synthesized from both alleles of the SNP, using allelic-quantitative PCR performed on heterozygous patients. Since rs12901499 is intronic, we could not use it directly in the allelic-quantitative PCR study. Instead, we used two transcript SNPs, rs3743342 and rs11556090, as proxy's for rs12901499. rs3743342 and rs11556090 are both located in the 3'UTR of SMAD3. We studied a total of 121 male and female patients, who had undergone either a hip or knee replacement. Linear regression was used to assess whether SMAD3 expression relative to genotype differed significantly from the null, whilst a 2-tailed Mann-Whitney exact test was used to assess the significance of any allelic differences.
Results: We found no evidence for a correlation between SMAD3 gene expression levels and genotype at the OA associated SNP rs12901499 in any of the tissues tested (all p-values were greater than 0.05). Since we had the genotype data available to us, we also stratified overall SMAD3 expression by genotype at rs3743342 and by genotype at rs11556090, the two 3'UTR SNPs that we had used. We were surprised to observe that genotype at these two SNPs did correlate with overall SMAD3 expression in cartilage, with a p-value of 0.0003 for rs3743342 and a p-value of 0.009 for rs11556090. In our allelic expression analysis we found no evidence that genotype at rs12901499 correlated with allelic expression imbalance. However, genotype at rs3743342 did correlate with allelic expression of SMAD3, with the A allele of the SNP producing, on average, 2.9 fold more transcript than the G allele (p-value less than 0.0001). When we tested this SNP for association in the arcOGEN GWAS of 7,410 OA cases and 11,009 controls, we observed moderate evidence of association in hip OA cases who had undergone total joint replacement, with a p-value of 0.001. It was the A allele of rs3743342, the allele that showed the greater expression in cartilage, which was more prevalent in OA cases.
Conclusions: In the tissues that we have studied our data does not support the OA association signal at SMAD3, which is marked by SNP rs12901499, as mediating an effect on the expression of the SMAD3 mRNA. We have however identified a polymorphism, rs3743342, which is located in the 3'UTR of SMAD3 and which does correlate with variation in the expression of the gene. rs3743342 also shows evidence, albeit moderate, of association with OA.

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GLOBAL GENE EXPRESSION OF DEDIFFERENTIATED CHONDROCYTES RELATIVE TO TISSUES OF MESENCHYMAL ORIGIN

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Purpose: It is now demonstrable that cells are not necessarily fated to terminal differentiation, but in the presence of a limited palette of transcription factors may be coerced towards an ostensibly pluripotent state. In models of regeneration cells within a regenerating blastema dedifferentiate, lose a functional, tissue phenotype, re-enter the cell cycle and express markers of earlier developmental stages. Chondrocytes in two-dimensional culture are well known to also lose their functional phenotype rapidly; progressive subculture alters the synthetic profile with loss of collagen type II and aggrecan hallmarks of this process. This is also termed 'dedifferentiation'. Common to both these situations is proliferation and a loss of differentiaton markers. The study hypothesised that the perturbations in functional phenotype following multiple passage is a consequence of differential expression of genes associated with an earlier developmental stage of cartilage development.
Methods: Tissue was obtained from 12 week old, male, F344 rats (n=10). This comprised of cartilage from the coxo-femoral and femoro-tibial joints (8 samples), tendon tissue from the tendon of the gastrocnemius muscle, deep digital flexor tendon and tail tendons (8 samples); dermal fibroblasts were obtained from flank skin (3 samples). Tissue was digested and cultured in standard growth medium, DMEM/10% foetal calf serum/1% penicillin-streptomycin. Monolayer cultures were expanded to 90% confluence and then sub-cultured at a 1:2 ratio for five occasions. In addition, native cartilage and tendon was harvested against which to compare dedifferentiated tissue (n=2). RNA was extracted using TriReagent (Ambion, Applied Biosciences) from 23 samples and this was amplified and labelled to produce biotin-labelled cRNA suitable for hybridisation to the Illumina RatRef-12 v1.0 BeadChip® array (Illumina, Inc., USA). Fluorescence emission was quantitatively detected using the Illumina iScan system. Raw bead-level intensity data was manipulated using the R programming platform and the 'Beadarray' open-source package. Differential expression analysis was undertaken using the lmFit and eBayes algorithms from the 'Limma' package. Results are presented as: log fold change, false discovery rate, and log odds ratio of differential expression.
Results: Hierarchical clustering of gene expression data from all 23 samples indicated that cell lines dedifferentiated in culture generated profiles that grouped them more closely to one another, rather than to their tissues of origin. Native cartilage was shown to cluster distant to dedifferentiated chondrocytes.

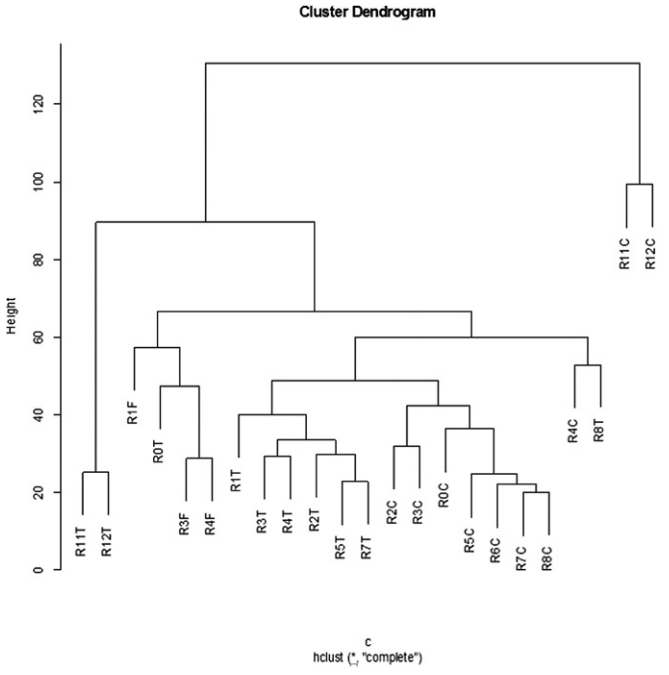

Figure 1. Native chondrocytes (R11C/12C) are strongly dissociated from dedifferentiated cartilage (R0C to R8C), which mostly cluster in a uniform group. Equally, dedifferentiated tenocytes (R0T to R8T) are weakly associated with their tissue of origin (R11/R12T). Cultured dermal fibroblasts (R1F to R4F) appear to group more closely with native tendon than dedifferentiated tenocytes. R4C and R8T are shown to group together separate from their specific culture groups. There was evidence of differential expression of the paired-type homeobox gene PITX1 in dedifferentiated chondrocytes, which is essential for cartilage development. Additionally TGFbeta2 and other markers of a pre-differentiated state were up-regulated.